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(54) Title: PRODUCTION OF PHOSPHOPEPTIDES FROM CASEIN			
(57) Abstract			
<p>A method for the preparation of selected phosphopeptides having anticariogenic and other activities, comprising the steps of completely digesting a soluble monovalent cation salt of casein in solution with a proteolytic enzyme, adding a mineral acid to the solution to adjust the pH to about 4.7, removing any precipitate produced, adding CaCl_2 to a level of about 1.0 % w/v to cause aggregation of at least the selected phosphopeptides in said digested solution, separating the aggregated phosphopeptides from the solution through a filter having a molecular weight exclusion limit lying substantially within the range 10,000 to 20,000 while passing the bulk of the remaining phosphopeptides and solution, diafiltrating the separated phosphopeptides with water through a filter and concentrating and drying the retentate.</p>			

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TITLE: PRODUCTION OF PHOSPHOPEPTIDES FROM CASEIN

Field of the Invention:

This invention relates to the production of phosphopeptides having anticariogenic and other properties from casein.

Background of the Invention:

In our Australian Patent No. 593365, we have described that four of the many phosphopeptides released by tryptic digestion of casein have anticariogenic (tooth-decay-inhibiting) activity. These peptides all contain the active sequence - Ser(P)-Ser(P)-Ser(P)-Glu-Glu- and correspond to α_1 (59-79) SEQ.ID No 2 (T_1), β (2-25) SEQ.ID No 3 (T_2), α_2 (46-70) SEQ.ID No 4 (T_4) and α_2 (2-21) SEQ.ID No 7 (T_3). The methods described for the production of the anticariogenic phosphopeptides are selective precipitation and ion exchange chromatography. While these methods produce very pure preparations of these peptides, they have not received general acceptance in the dairy industry due to their cost and the level of technical skill required.

Recently membrane ultrafiltration has found broad acceptance in the dairy industry for milk treatment. In U.S. Patents 4,358,465, 4,361,587 and 4,495,176, Brule et al describe an ultrafiltration method for the

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production of casein phosphopeptides as dietetic aliments. This procedure proves unsuitable for the production of anticariogenic phosphopeptides due to the predominance of non-anticariogenic phosphopeptides in the preparations.

Summary of the Invention and Object:

It is an object of the present invention to provide a method of preparing selected phosphopeptides from casein using ultrafiltration.

The invention provides a method for the preparation of selected phosphopeptides comprising the steps of completely digesting a soluble monovalent cation salt of casein in solution, introducing a di or trivalent metal ion to cause aggregation of at least the selected phosphopeptides in said digested solution, and filtering the solution through a filter having a molecular weight exclusion limit selected to retain at least said aggregated phosphopeptides while passing the bulk of the remaining phosphopeptides.

In the method described by Brule et al, the object is to obtain a broad range of phosphopeptides from casein for use as a dietetic aliment. Therefore Brule et al do not teach that the casein compound must be completely hydrolysed or that a di/trivalent metal ion must be added to aggregate the desired phosphopeptides to a size which enables those

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phosphopeptides to be filtered from the solution while allowing the remaining phosphopeptides and solution to pass. This represents a significant advance in the art since it enables the use of an industry accepted method of extraction which results in a preparation which is rich (> 90% w/w) in the desired phosphopeptides.

In a preferred form of the invention, the selected phosphopeptides are the anticariogenic phosphopeptides referred to above, and the molecular exclusion limit adopted during the filtering step of the above method preferably substantially falls within the range 10,000 to 20,000.

The soluble monovalent cation salt of casein, such as sodium caseinate or potassium caseinate, may be present in the solution in a concentration substantially falling within the range 0.1 to 50% w/w, which is preferably digested using a proteolytic enzyme, such as pancreatin, trypsin, papain or chymotrypsin, or a mixture of proteolytic enzymes such as trypsin and chymotrypsin or by chemical means, such as cyanogen bromide. The enzyme(s) to casein ratio can range from about 1:1000 to 1:10 (w:w) but this would be selected to allow complete digestion of the casein as defined above. The pH of the hydrolysis should preferably be controlled at optimum for the enzymes to

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allow complete casein digestion. The temperature also should be optimised for complete digestion but temperature induced degradation (deamidation, dephosphorylation and peptidolysis) should be minimised. The optimal temperature is between about 20°C and 60°C.

In a preferred form of the invention, after digestion HCl is added at room temperature to about pH 4.7 and any precipitate (this should be minimal) removed. CaCl_2 is then added to the supernatant to a level of about 1.0% w/v. Phosphopeptides in the presence of 1.0% w/v calcium (II) aggregate. The anticariogenic phosphopeptides (ie. containing the sequence -Ser(P)-Ser(P)-Ser(P)-Glu-Glu-) form hexamers which are separated from the smaller non-anticariogenic phosphopeptide aggregates by extensive diafiltration through a 10,000 molecular weight exclusion limit filter with a CaCl_2 solution preferably 1.0% w/v. The preferred molecular weight exclusion limit of the membrane filter should not be less than 10,000 or greater than about 20,000. The addition of a CaCl_2 solution, or some other suitable di/trivalent metal ion, such as zinc (II) or ferric (III), is essential for diafiltration in order to maintain the integrity of the anticariogenic phosphopeptide aggregates thus allowing separation of the

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anticariogenic from the non-anticariogenic phosphopeptides.

After several volumes of 1.0% CaCl₂ w/v have passed through the membrane filter to achieve greater than 90% purity of the anticariogenic phosphopeptides the ultraretentate containing the anticariogenic phosphopeptides can be diafiltered with water through a 1,000 molecular weight exclusion limit filter to remove calcium if desired. The retentate is then concentrated and spray dried.

The calcium, zinc and ferric salts of the anticariogenic phosphopeptide preparation (ACPP) can be converted to a sodium salt by acidifying a 10% w/v solution of the calcium ACPP to a low pH, circa pH 2.0, with HCl. After extensive diafiltration through a 1,000 molecular weight exclusion limit filter the retentate is neutralised to pH 7.0 with NaOH and then diafiltrated with water through the same filter to remove excess sodium chloride.

The calcium ACPP can be converted to calcium phosphate ACPP by addition of CaCl₂ and Na₂HPO₄ where the Ca/P final ratio is 1.67. The peptide $\alpha_{11}(59-79)$ can bind 21 Ca and 13 PO₄. The filtrate of the above process is suited for the purification of other bioactive casein peptides by size and charge-based separation technologies and can be used as

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microbiological growth media, as dietary supplements after debittering or as a nitrogen fertilizer.

A presently preferred embodiment of the invention will now be described with reference to the following example.

EXAMPLE

Sodium caseinate was prepared by acidifying milk with 0.1 M HCl to pH 4.7 and neutralising the precipitate with NaOH to pH 7.0. A 10% w/v solution of sodium caseinate was prepared and adjusted to pH 8.0. Trypsin (Novo) was added to 0.2% w/v and the hydrolysis allowed to proceed to completion at 50°C with adjustment to pH 8.0 by constant addition of NaOH. The pH of the solution was then adjusted to pH 4.7 with 5 M HCl and the precipitate removed at room temperature by centrifugation. The supernatant was microfiltered through an 8 micron filter, and then adjusted to pH 7.0 with NaOH and CaCl₂, added to a level of 1.0% w/v. This solution was then diafiltered through an Amicon YM10 (10,000 molecular weight exclusion limit) with 3 to 5 volumes of 1.0% w/v CaCl₂. The retentate was then washed with 1 volume of distilled/deionised water through an Amicon YM1 filter (1,000 molecular weight exclusion limit). The individual peptides of this preparation were separated by ion exchange FPLC and reverse phase HPLC as described in the aforementioned patent and identified

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by amino acid composition and sequence analyses after conversion of the Ser(P) residues to S-ethyl cysteine. An analysis of the preparation is shown in Table 1.

Table 1. Composition of an Anticariogenic Phosphopeptide Preparation

<u>Peptide*</u>	<u>w/w</u>
α_{s2} (1-21) (SEQ.ID No 8)	0.8
B(1-25) (SEQ.ID No 1)	22.3
α_{s2} (2-21) (SEQ.ID No 7) (T ₃)	5.7
B(2-25) (SEQ.ID No 3) (T ₁)	17.9
α_{s1} (59-79) (SEQ.ID No 2) (T ₁)	21.4
Desamido ["] α_{s1} (59-79) (SEQ.ID No 5)	6.3
α_{s2} (46-70) (SEQ.ID No 4) (T ₄)	6.8
Desamido ["] α_{s1} (59-79) (SEQ.ID No 6)	6.4
α_{s1} (43-79) (SEQ.ID No 9)	3.3
NAP*	9.1

* NAP = non-anticariogenic peptides

This preparation contains the four anticariogenic phosphopeptides described in the aforementioned patent, [B(2-25), T₁, α_{s1} (59-79), T₁, α_{s2} (2-21), T₃ and α_{s2} (46-70), T₄], those related peptides incompletely hydrolysed by trypsin [α_{s2} (1-21), B(1-25) and α_{s1} (43-79)] and also minor levels of the two deamidated forms of α_{s1} (59-79), desamido["] and desamido["]["] which result from temperature induced deamidation, this occurs in

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an even greater extent in commercial production of sodium caseinate due to higher temperatures and extremes of pH, although the presence of the deamidated forms has no effect on anticariogenic activity. The anticariogenic phosphopeptides were 90.9% w/w of the peptides produced.

If pure α_1 -casein is used in place of casein then $\alpha_1(59-79)$ will be obtained by this process with minor amounts of the deamidated forms of this peptide depending on hydrolysis conditions. If pure β -casein is used then only $\beta(1-25)$ and $\beta(2-25)$ will be obtained using this process.

When crude enzymes are used (such as pancreatin), slight truncation (both N- and C- terminally) of the nine listed phosphopeptides can occur. As long as this truncation is only slight, there is no loss of activity. In fact, pancreatin produces a preparation with slightly greater specific activity on a weight basis when compared with purified trypsin.

The sequences of the nine peptides, which include the peptides T_1 to T_4 of the aforementioned patent, and the other peptides referred to above are detailed in the following sequence listing

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: REYNOLDS, ERIC CHARLES
(ii) TITLE OF INVENTION: PRODUCTION OF PHOSPHOPEPTIDES FROM CASEIN
(iii) NUMBER OF SEQUENCES: 9

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE:
(B) STREET:
(C) CITY:
(D) STATE:
(E) COUNTRY:
(F) ZIP: 7

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: FLOPPY DISK
(B) COMPUTER: IBM PC COMPATIBLE
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: WORD PERFECT

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A)
(B)
(C)

(ix) TELECOMMUNICATION INFORMATION:

(A)
(B)
(C)

(2) INFORMATION FOR SEQ.ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25
(B) TYPE: Amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Protein

(ix) FEATURE:

(A) NAME/KEY: Phosphoserine
(B) LOCATION: 15
(D) OTHER INFORMATION:
Post-translationally phosphorylated serine

(ix) FEATURE:

(A) NAME/KEY: Phosphoserine
(B) LOCATION: 17
(D) OTHER INFORMATION:
Post-translationally phosphorylated serine

(ix) FEATURE:

(A) NAME/KEY: Phosphoserine
(B) LOCATION: 18
(D) OTHER INFORMATION:

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(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Protein
(ix) FEATURE:
(A) NAME/KEY: Phosphoserine
(B) LOCATION: 14
(D) OTHER INFORMATION:
Post-translationally phosphorylated serine
(ix) FEATURE:
(A) NAME/KEY: Phosphoserine
(B) LOCATION: 16
(D) OTHER INFORMATION:
Post-translationally phosphorylated serine
(ix) FEATURE:
(A) NAME/KEY: Phosphoserine
(B) LOCATION: 17
(D) OTHER INFORMATION:
Post-translationally phosphorylated serine
(ix) FEATURE:
(A) NAME/KEY: Phosphoserine
(B) LOCATION: 18
(D) OTHER INFORMATION:
Post-translationally phosphorylated serine

(xi) SEQUENCE DESCRIPTION: SEQ.ID NO:3:

Glu Leu Glu Glu Leu Asn Val Pro Gly Glu Ile Val Glu Ser Leu Ser
1 5 . 10 15

Ser Ser Glu Glu Ser Ile Thr Arg
20

(2) INFORMATION FOR SEQ.ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25
(B) TYPE: Amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Protein

(ix) FEATURE:
(A) NAME/KEY: Phosphoserine
(B) LOCATION: 11
(D) OTHER INFORMATION:
Post-translationally phosphorylated serine

(ix) FEATURE:
(A) NAME/KEY: Phosphoserine
(B) LOCATION: 12
(D) OTHER INFORMATION:
Post-translationally phosphorylated serine

(ix) FEATURE:
(A) NAME/KEY: Phosphoserine
(B) LOCATION: 13
(D) OTHER INFORMATION:
Post-translationally phosphorylated serine

(ix) FEATURE:
(A) NAME/KEY: Phosphoserine
(B) LOCATION: 15
(D) OTHER INFORMATION:
Post-translationally phosphorylated serine

(xi) SEQUENCE DESCRIPTION: SEQ. ID NO:4:

Asn Ala Asn Glu Glu Glu Tyr Ser Ile Gly Ser Ser Ser Glu Glu Ser
 1 5 10 15

Ala Glu Val Ala Thr Glu Glu Val Lys
20 25

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(2) INFORMATION FOR SEQ.ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21

(B) TYPE: Amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Protein

(ix) FEATURE:

(A) NAME/KEY: Phosphoserine

(B) LOCATION: 6

(D) OTHER INFORMATION:
Post-translationally phosphorylated serine

(ix) FEATURE:

(A) NAME/KEY: Phosphoserine

(B) LOCATION: 8

(D) OTHER INFORMATION:
Post-translationally phosphorylated serine

(ix) FEATURE:

(A) NAME/KEY: Phosphoserine

(B) LOCATION: 9

(D) OTHER INFORMATION:
Post-translationally phosphorylated serine

(ix) FEATURE:

(A) NAME/KEY: Phosphoserine

(B) LOCATION: 10

(D) OTHER INFORMATION:
Post-translationally phosphorylated serine

(ix) FEATURE:

(A) NAME/KEY: Phosphoserine

(B) LOCATION: 17

(D) OTHER INFORMATION:
Post-translationally phosphorylated serine

(xi) SEQUENCE DESCRIPTION: SEQ. ID NO:5:

Gln Met Glu Ala Glu Ser Ile Ser Ser Ser Glu Glu Ile Val Pro Asp
1 5 10 15

Ser Val Glu Gln Lys
20

(2) INFORMATION FOR SEQ.ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21

(B) TYPE: Amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Protein

(ix) FEATURE:

(A) NAME/KEY: Phosphoserine

(B) LOCATION: 6

(D) OTHER INFORMATION:
Post-translationally phosphorylated serine

(ix) FEATURE:

(a) Name/Key: Phosphoserine

(b) Location: 8

(d) Other information:
Post-translationally phosphorylated serine

(ix) Feature:

(a) Name/Key: Phosphoserine

(b) Location: 9

(d) Other information:
Post-translationally phosphorylated serine

(ix) Feature:

(a) Name/Key: Phosphoserine

(b) Location: 10

(d) Other information:

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Post-translationally phosphorylated serine

(ix) Feature:
 (a) Name/Key: Phosphoserine
 (b) Location: 17
 (d) Other information:
 Post-translationally phosphorylated serine

(xi) SEQUENCE DESCRIPTION: SEQ.ID NO:6:

Gln Met Glu Ala Glu Ser Ile Ser Ser Glu Glu Ile Val Pro Asp
 1 5 10 15

Ser Val Glu Glu Lys
 20

(2) INFORMATION FOR SEQ.ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20
 (B) TYPE: Amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Protein

(ix) Feature:

(a) Name/Key: Phosphoserine
 (b) Location: 7
 (d) Other information:
 Post-translationally phosphorylated serine

(ix) Feature:

(a) Name/Key: Phosphoserine
 (b) Location: 8
 (d) Other information:
 Post-translationally phosphorylated serine

(ix) Feature:

(a) Name/Key: Phosphoserine
 (b) Location: 9
 (d) Other information:
 Post-translationally phosphorylated serine

(ix) Feature:

(a) Name/Key: Phosphoserine
 (b) Location: 15
 (d) Other information:
 Post-translationally phosphorylated serine

(xi) SEQUENCE DESCRIPTION: SEQ.ID NO:7:

Asn Thr Met Glu His Val Ser Ser Glu Glu Ser Ile Ile Ser Gln
 1 5 10 15

Glu Thr Tyr Lys
 20

(2) INFORMATION FOR SEQ.ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
 (B) TYPE: Amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Protein

(ix) Feature:

(a) Name/Key: Phosphoserine
 (b) Location: 8
 (d) Other information:
 Post-translationally phosphorylated serine

(ix) Feature:

(a) Name/Key: Phosphoserine
 (b) Location: 9
 (d) Other information:

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Post-translationally phosphorylated serine

(ix) FEATURE:

- (a) Name/Key: Phosphoserine
- (b) Location: 10
- (d) Other information:
Post-translationally phosphorylated serine

(ix) FEATURE:

- (a) Name/Key: Phosphoserine
- (b) Location: 16
- (d) Other information:
Post-translationally phosphorylated serine

(xi) SEQUENCE DESCRIPTION: SEQ.ID NO:8:

Lys Asn Thr Met Glu His Val Ser Ser Ser Glu Glu Ser Ile Ile Ser
1 5 10 15

Gln Glu Thr Tyr Lys
20

(2) INFORMATION FOR SEQ.ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 37
- (B) TYPE: Amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Protein

(ix) FEATURE:

- (A) NAME/KEY: Phosphoserine
- (B) LOCATION: 4
- (D) OTHER INFORMATION:
Post-translationally phosphorylated serine

(ix) FEATURE:

- (A) NAME/KEY: Phosphoserine
- (B) LOCATION: 6
- (D) OTHER INFORMATION:
Post-translationally phosphorylated serine

(ix) FEATURE:

- (A) NAME/KEY: Phosphoserine
- (B) LOCATION: 22
- (D) OTHER INFORMATION:
Post-translationally phosphorylated serine

(ix) FEATURE:

- (A) NAME/KEY: Phosphoserine
- (B) LOCATION: 24
- (D) OTHER INFORMATION:
Post-translationally phosphorylated serine

(ix) FEATURE:

- (A) NAME/KEY: Phosphoserine
- (B) LOCATION: 25
- (D) OTHER INFORMATION:
Post-translationally phosphorylated serine

(ix) FEATURE:

- (A) NAME/KEY: Phosphoserine
- (B) LOCATION: 26
- (D) OTHER INFORMATION:
Post-translationally phosphorylated serine

(ix) FEATURE:

- (A) NAME/KEY: Phosphoserine
- (B) LOCATION: 33
- (D) OTHER INFORMATION:
Post-translationally phosphorylated serine

(xi) SEQUENCE DESCRIPTION: SEQ.ID NO:9:

Asp Ile Gly Ser Glu Ser Thr Glu Asp Gln Ala Met Glu Asp Ile Lys
1 5 10 15

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Gln Met Glu Ala Glu Ser Ile Ser Ser Ser Glu Glu Ile Val Pro Asn
20 25 30

Ser Val Glu Gln Lys
35

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CLAIMS

1. A method for the preparation of selected phosphopeptides comprising the steps of completely digesting a soluble monovalent cation salt of casein in solution, introducing a di or trivalent metal ion to cause aggregation of at least the selected phosphopeptides in said digested solution, and filtering the solution through a filter having a molecular weight exclusion limit selected to retain at least said aggregated phosphopeptides while passing the bulk of the remaining phosphopeptides in a filtrate.
2. The method of claim 1, wherein the selected phosphopeptides are anticariogenic phosphopeptides and the molecular weight exclusion limit adopted during the filtering step substantially falls within the range 10,000 to 20,000.
3. The method of claim 1 or 2, wherein the soluble monovalent cation salt of casein is present in the solution in a concentration substantially falling within the range 0.1 to 50% w/w.
4. The method of any one of claims 1 to 3, wherein the digestion step is performed using a proteolytic enzyme and the ratio of proteolytic enzyme to soluble monovalent cation salt of casein in the solution falls substantially within the range 1:1000 to 1:10 (w:w)

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selected to allow complete digestion of the casein salt.

5. The method of any preceding claim, wherein the pH and the temperature of the solution is controlled to allow complete digestion of the casein salt.

6. The method of claim 5, wherein the temperature of the solution lies substantially within the range 20°C to 60°C.

7. A method for the preparation of selected phosphopeptides having anticariogenic and other activities, comprising the steps of completely digesting a soluble monovalent cation salt of casein in solution with a proteolytic enzyme, adding a mineral acid to the solution to adjust the pH to about 4.7, removing any precipitate produced, adding CaCl₂ to a level of about 1.0% w/v to cause aggregation of at least the selected phosphopeptides in said digested solution, separating the aggregated phosphopeptides from the solution through a filter having a molecular weight exclusion limit lying substantially within the range 10,000 to 20,000 while passing the bulk of the remaining phosphopeptides and solution in a filtrate, diafiltrating the separated phosphopeptides with water through a filter and concentrating and drying the retentate.

8. The method of claim 1 or 7, wherein the soluble

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monovalent cation salt of casein is selected from sodium caseinate and potassium caseinate.

9. The method of claim 7 or 8, wherein proteolytic enzyme is selected from pancreatin, trypsin, papain, chymotrypsin or mixtures thereof.

10. A method for the preparation of anticariogenic phosphopeptides substantially as hereinbefore described with reference to the specific example.

11. Phosphopeptides when produced by the method of any preceding claim.

12. The filtrate of the method of any one of claims 1 to 10.

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)⁸

According to International Patent classification (IPC) or to both National Classification and IPC
Int. Cl.⁸ C07K 3/02, 7/10, 15/24, C12P 21/06, A23J 1/22, A23J 3/10

II. FIELDS SEARCHED

Minimum Documentation Searched⁷

Classification System	Classification Symbols
IPC ⁵	C07K 3/02, 7/10, 15/24, C12P 21/06, A23J 1/22, 3/10
IPC ³	C07C 103/52

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched⁸

AU: IPC as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹

Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate of the relevant passages ¹²	Relevant to Claim No ¹³
X	Patent Abstracts of Japan, C-260, page 49, JP, A, 59-159793 (MEIJI SEIKA K.K.) 28 February, 1983 (28.02.83).	1 to 6
X	Patent Abstracts of Japan, C261, page 66, JP, A, 59-162843 (MEIJI SEIKA K.K.) 8 March, 1983 (08.03.83).	1 to 6
X	Derwent Abstract Accession no. 76-58968X/31, Class D16, JP, A, 51-070888 (IDEMITSU KOSAN) 18 June, 1976 (18.06.76).	1

(continued)

• Special categories of cited documents : ¹⁰		
"A" Document defining the general state of the art which is not considered to be of particular relevance	"T"	Later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier document but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means		
"P" document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search 6 July 1992 (06.07.92)	Date of Mailing of this International Search Report 16 July 1992 (16.07.92)
International Searching Authority AUSTRALIAN PATENT OFFICE	Signature of Authorized Officer A. BESTOW 

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document, ¹¹ with indication, where appropriate of the relevant passages ¹²	Relevant to Claim No ¹³
X	EP, A, 0090406 (MEIJI SEIKA KAISHA LTD) 5 October, 1983 (05.10.83)	1 to 6
A	See page 19, line 6 to page 22, line 1 and page 8, line 8-17.	7 to 12
X	AU, B, 51491/85 (600225) (ROUSSEL UCLAF) 12 June 1986 (12.06.86). See page 9, line 6 to page 18, line 22.	1 to 12
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ANNEX TO THE INTERNATIONAL SEARCH REPORT ON
INTERNATIONAL APPLICATION NO. PCT/AU 92/00175

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

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END OF ANNEX